

Influence of Sex Ratio and Density on the Comparative Repellency of IR3535[®] Against Caged *Aedes*, *Anopheles*, and *Culex* Mosquitoes

SILVANA KITADAI NAKAYAMA, RÜEDIGER GRAF,
ANJA-MARTINA BOHLMANN, JUTTA ZURLAGE,
HOWARD EPSTEIN, MÁRCIA R.T. DE CAMARGO RANZANI,
and FRANK PFLÜECKER, Merck S.A., 064565-130 São Paulo,
Brazil (S.K.N.), Department of Chemical Engineering and Biotechnology,
University of Applied Sciences Darmstadt, 64239, Darmstadt, Germany
(R.G.), Merck KGaA, Performance Materials, 64293 Darmstadt, Germany
(A.-M.B., J.Z., F.P.), EMD Performance Materials Corp., Philadelphia, PA
(H.E.), Bioagri Institute, 13515-000 São Paulo, Brazil (M.R.T.de.C.)

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Synopsis

Studies on topical repellent efficacy conducted with caged mosquitoes in the laboratory are important to both the development and regulation of insect repellents. Guidelines for laboratory studies stipulate specific densities, sex ratios, and biting rates, whereas those for field studies are governmentally required before a promising repellent can be registered for human use. These protocols stipulate minimum biting rates alone. Relatively little is known, however, about the influence of mosquito density and sex ratio on their biting propensity, either in the field or laboratory. Using Environmental Protection Agency guidelines for cage testing, we studied the influence of mosquito density and sex ratio in laboratory repellency tests of the biopesticide Ethyl butylacetylaminopropionate (IR3535[™]) (20%) against three mosquito species (*Aedes aegypti*, *Anopheles aquasalis*, and *Culex quinquefasciatus*). DEET [3-(*N*-acetyl-*N*-butyl) aminopropionic acid ethyl ester] (20%) in a laboratory prepared formulation was used as a comparison article. Studies were conducted by trained investigators at the BioAgri Laboratories in Brazil. We found that higher mosquito density generally decreased protection time, but that the influence of sex ratio was more complex. The presence of male mosquitoes increased protection times against *Aedes* and *Anopheles* perhaps because mate-seeking males interfered with female feeding. Interestingly, by contrast, protection times decreased against *Culex* in the presence of males. Such considerations may potentially assist in improving the match between cage and field testing under a broader range of conditions that permit more accurate labeling of repellents for safe and effective use by consumers.

INTRODUCTION

Mosquito repellents are important for personal protection against nuisance biting and mosquito-transmitted pathogens, including those that cause malaria, dengue fever, and

Address all correspondence to Howard Epstein at howard.epstein@emdgroup.com.

West Nile fever (1). The protection a repellent affords is influenced by numerous factors, including attributes of the insects, hosts, environmental conditions, repellent formulation, and dosing, and the interactions of these factors are reviewed by Barnard (2) and Carroll (3). Many of these factors have been studied with laboratory strains of caged female mosquitoes; however, no general consensus has been reached regarding the optimal mosquito biting rate and density for cage-based repellency tests (2). Indeed, contemporary cage-testing guidelines issued by regulatory arms of federal and international public health agencies differ substantially when reviewing the U.S. Environmental Protection Agency (EPA) (4), the European Union (5), and the World Health Organization (6) guidelines.

In a complementary approach, the performance of repellent formulations that prove promising in the laboratory is often verified under expected conditions of use in nature with wild mosquito populations. In that circumstance, some of the factors that influence efficacy are not controlled, permitting natural variation. Uncontrolled variables include environmental conditions and mosquito species demography. Furthermore, field tests frequently include more human subjects than are feasible in laboratory tests, better representing the range of people to be protected. Review of submitted data from field tests is required as part of required vetting for permission to market labeled repellents by the U.S. EPA and E.U. Biocides Division, and likewise recommended by the World Health Organization (6).

Factors that may influence repellent performance in nature, including population density, sex ratio, and age structure, can readily be measured in the laboratory. Biological factors that have been shown by cage studies to influence the efficacy of topical mosquito repellents include larval diet, carbohydrate availability to adult mosquitoes, age and reproductive history of adult female mosquitoes, partial blood engorgement, and inherent qualities of repellent-treated test subjects (7–9). Biting patterns can also vary with the size of the cage and density (7,8,10). Sex ratio influences have not been quantified, although excluding males from laboratory tests to control interference with females is conventional. Given that males are naturally present in wild populations, a more systematic approach to laboratory studies of sex ratio and repellency is needed. Although laboratory tests allow controlled conditions to aid identification of new repellents for field evaluation (2), sex ratio and its interplay with population density are understudied factors for which laboratory testing remains warranted.

Cage-based laboratory testing of mosquito repellents is the standard approach for screening actives and formulations before and during product development, because it is more convenient, is lower in cost than field testing and repeatable in the laboratory setting. These studies are also suitable for comparing performance among marketed repellent products for consumer education. Cage testing allows environmental variables to be controlled and manipulated; on the other hand, such testing does not match typical conditions of repellent use by consumers. Given the reciprocal liabilities of laboratory and field studies, an additional study of factors influencing laboratory performance offers potential to improve the design and interpretation of insect repellent development studies and suggests factors that may be important to the design of field studies and laboratory cage studies. Currently, the best approach to field and laboratory testing is to conduct testing with as many individuals as possible.

In this study, we examine the effects of sex ratio and density on repellent performance against three genera of mosquitoes under caged conditions. This study provides data for

a non-DEET molecule, registered by the Environmental Protection Agency as a biopesticide repellent IR3535[®] [3-(*N*-acetyl-*N*-butyl) aminopropionic acid ethyl ester]. We used DEET as a positive control. The aim of the study was to examine those factors and further understand the dynamics in the influence of the study outcome by comparison of the repellent efficacy of IR3535[®] with that of DEET. If indeed sex ratio and density have an influence on study outcome, this might provide a rationale guiding future revisions of label instructions that provide safe and effective consumer use.

MATERIALS AND METHODS

This study was conducted by BioAgri Laboratories, Bela Vista, Charqueada, Sao Paulo, Brazil. Testing was conducted in two periods. A group of five volunteers participated in tests with *Aedes* mosquitoes in June 2011, and a second group of five volunteers participated in tests with *Anopheles* and *Culex* mosquitoes in October and November 2011. Testing in these two periods was conducted under similar environmental conditions as detailed in the section Exposures to Mosquitoes. Only very broad comparisons are made between species; this reduces the risk that temporal division confounds important interpretations. Testing was conducted using an EPA protocol. Test materials were applied to volunteers by trained technicians at BioAgri Laboratories.

REPELLENTS

The test repellent formulation consisted of a hydroalcoholic pump-spray containing 20% IR3535[®] with 2% of a film-forming agent, a PVP/VA copolymer—C₁₀H₁₅NO₃, added to the product to improve efficacy. The comparison article was a commercial 20% DEET in 80% ethyl alcohol formulation. Both test materials were prepared in the laboratory of Merck KGaA (Darmstadt, Germany) in Brazil.

IR3535[®] (Figure 1) was developed by Merck KGaA in 1975, and has been marketed in Europe for more than 20 years and elsewhere in recent years. Its mechanism of repellency is similar to that of DEET. IR3535[®] acts by forming a vapor barrier surface on skin that inhibits feeding by certain blood-feeding arthropods (11). IR3535[®] was registered by the U.S. EPA in 1999 as a biopesticide because it is functionally identical to naturally occurring beta-alanine. IR3535[®] is a substituted β-amino acid that contains 98% 3-(*N*-acetyl-*N*-butyl) aminopropionic acid ethyl ester as the active ingredient and 2% inert ingredients. Biopesticides receive special consideration by EPA because they are generally considered to be less toxic and are more specific than conventional pesticides (12,13). Registered IR3535[®] formulations are labeled as repellents against mosquitoes, deer ticks, body lice, and biting flies globally depending on the registration in specific countries.

The repellent efficacy of IR3535[®] has been investigated in numerous field and laboratory tests around the world against a wide variety of arthropods. Efficacy is shown to vary for similar concentrations of active ingredients (14–17) perhaps in part because of differences in formulation (emulsion, spray, or lotion). IR3535[®] performs similarly to DEET when tested against mosquitoes and ticks using comparable emulsion systems and concentrations (18).

Mean CPT (min)

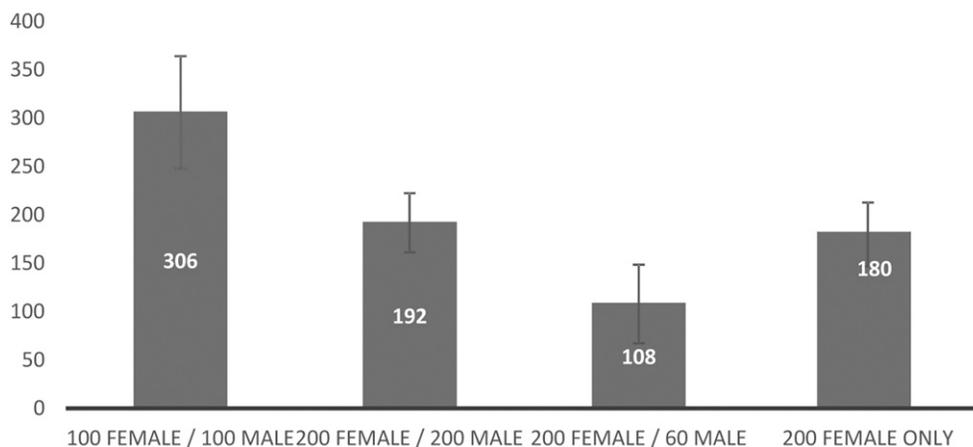


Figure 1. Repellent activity of 20% IR3535(R) against *Aedes aegypti* in a cage test with 5 volunteers, shown as mean complete protection time.

HUMAN TEST SUBJECTS

Volunteer recruitment was made by BioAgri Laboratories. During subjects' selection for the study, the physician in charge certified that the subjects have no pathologies that might interfere with the study results. The physician was also responsible for all information contained in the subject's evaluation form, by checking all inclusion and noninclusion criteria for admission of each subject in the study. The study was conducted according to the recommendations of the National Council of Health, Resolution number 196/96, an Institutional Review Board (IRB) of Brazil.

Each experiment was carried out with five healthy test subjects of both genders, between 18 and 65 years of age. Each subject stated that he or she had either no or slight dermal irritation when bitten by mosquitoes. Subjects read information sheets and signed informed consent forms before participating in the study. They agreed not to use scented cosmetic products or ingest alcohol or caffeine beginning 12 h before the test.

Subject exclusion criteria included attractiveness to mosquitoes. This criterion was assessed in a preliminary exposure in which each candidate exposed a bare, untreated forearm in an individual cage containing the mosquitoes. Mosquito species tested were *Aedes*, *Culex*, and *Anopheles*, using different sex ratios: 100 females:100 males; 200 females:200 males; 200 females:60 males; and 200 females:0 male (female only), for a period of 30 s. Volunteers who showed natural repellency, defined as no landings, were excluded from the test (Figure 1).

MOSQUITO SPECIES IN THE STUDY

Three mosquito species were evaluated: *Aedes aegypti*, *Culex quinquefasciatus*, and *Anopheles aquasalis*. *Ae. aegypti* is important in the transmission of pathogens that cause dengue fever, yellow fever, and chikungunya. *Cx. quinquefasciatus* vectors West Nile fever and equine

encephalitis pathogens. *An. aquasalis* is an important vector of the malaria pathogen *Plasmodium vivax* in coastal regions of the neotropics. All mosquitoes were reared in the laboratory at 23–27°C and 50–70% relative humidity and in a 12:12 light-to-dark cycle. On eclosion, the new adults were segregated by gender and maintained on a 10% sucrose solution in water. Mosquitoes used in the study were obtained from a population maintained in the laboratory.

APPLICATION OF TEST MATERIALS

Forearm dimensions of qualified subjects were measured and entered into the following formula to calculate the quantity of test material needed by each to receive a dosage of 1 g per 600 cm² of forearm skin:

$$\text{Amount [g]} \approx \frac{M1 > M2 > M3 > M4}{4} \frac{L}{600}$$

Here, L = forearm length [cm], M1 = wrist circumference [cm], M4 = elbow circumference [cm], and M2 and M3 = two equally distant forearm circumference measures from the wrist to elbow [cm²].

Subjects prepared for repellent application by first washing their forearms with soap, followed by a thorough rinse with water and drying with paper towels. Subjects were then supplied with the calculated amount of the test product. While protecting the hands with rubber gloves, a technician evenly spread the supplied test product on one of the forearms of each volunteer. All test subjects were directed not to touch or rub the treated arm between evaluation periods.

EXPOSURES TO MOSQUITOES

The mosquitoes used for testing were 5- to 10-d-old, disease-free, adult males and females. Both sexes were virgin, and the females had no prior opportunity to blood-feed. On each test day, they were released in groups by species into individual metal-screened cages (61 × 61 × 61 cm³). Test conditions were adjusted to account for the biting behavior of the species tested. For *Ae. aegypti*, the room was kept illuminated with temperature at 25° ± 2°C and humidity at 50–70%. For *Cx. quinquefasciatus*, the room was kept dark with infrared illumination from the top to the bottom of the cage, or laterally during the test. This species bites at night, and temperature was set for 25° ± 2°C with humidity between 50% and 70%. For *An. aquasalis*, the illumination was penumbra, partial light, with relative humidity not lower than 70% and temperature between 24° and 28°C.

Across four treatments, the total number of mosquitoes per cage was varied between 200 and 400, and the female: male ratio was varied between 1:1 and 1:0 (Figure 1). IR3535® was tested against all three mosquito species at all densities and sex ratios. The DEET positive control was tested solely using 200 females (no males) per cage. Females-only is the standard configuration used for cage-based testing of mosquito repellent efficacy.

Tests were conducted at 23–27°C and >70% RH. Beginning approximately 30 min after test materials were applied, subjects inserted their treated forearms individually through

a voile sleeve into the test cage and exposed them to the mosquitoes for 5 min, after which the arms were withdrawn from the cage. Reexposure occurred every 30 min. The test ended for an individual subject when there was a confirmed bite. A confirmed bite was defined as the occurrence of more than one bite within the same exposure period (in this case, a period of 30 min) or when one bite was followed by another in a consecutive exposure, which confirmed the result of the previous period. The number of landings was also recorded until the end of the test.

DATA SCORING, TABULATION, AND ANALYSIS

The duration of repellency for each subject in each exposure was calculated as the time between test product application and confirmed bite. This duration is the complete protection time (CPT). Mean CPT (± 1 standard deviation) was determined for each test subject.

RESULTS

Influence of density and sex ratio on CPT by IR3535[®].

Ae. AEGYPTI

The sex ratio and density manipulations strongly influenced CPT in *Ae. aegypti* ($p < 0.003$; Figure 2). CPT averaged approximately 3 h or slightly longer when tested against 200 females alone or 200 females:200 males and was almost halved at the 3.3:1 ratio

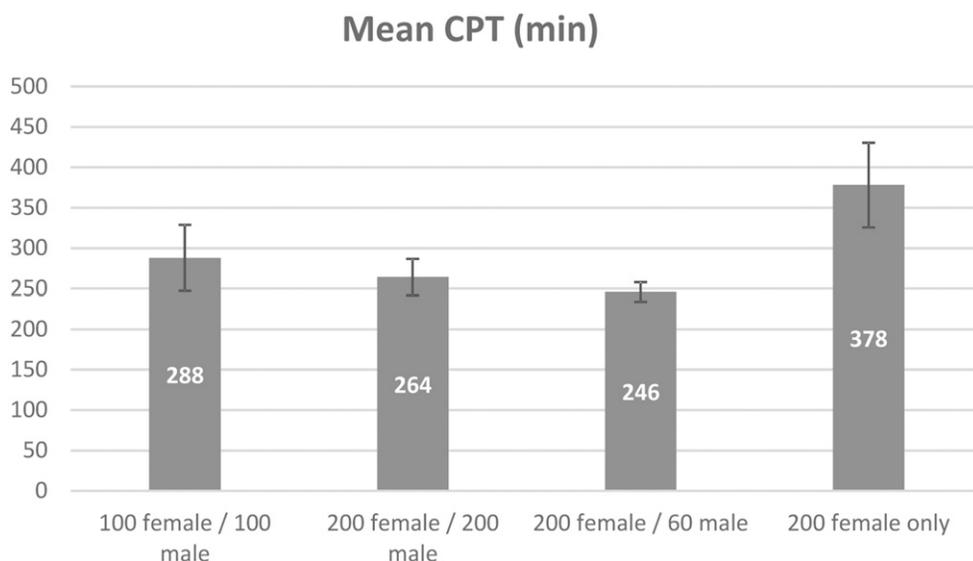


Figure 2. Repellency of 20% IR3535[®] against *Culex quinquefasciatus* as cage test. The values of five volunteers are shown as mean CPT \pm standard deviation.

(200 female:60 males). CPT with 100 females:100 males was significantly longer than that in the other treatments, averaging greater than 5 h, whereas CPT with 200 females:60 males was significantly shorter than that in all other treatments (Figure 2).

CX. QUINQUEFASCIATUS

CPTs against *Cx. quinquefasciatus* (Figure 3) averaged longer in all treatments than that against the other species and were likewise influenced by the treatment ($p < 0.01$). Unlike the *Aedes* and *Anopheles* trials, *Culex* CPT was longest in the absence of males ($p < 0.01$). This suggests a possible male facilitation or stimulation of, rather than net interference with, female feeding. IR3535[®] was comparable to DEET (as presented in the Repellents section): it provided complete protection for approximately 6 h in the absence of males. CPTs dropped significantly to between 4 and 5 h in the other treatments, with the shortest duration at the 3.3:1 sex ratio for *Ae. aegypti*.

AN. AQUASALIS

The results for *An. aquasalis* are given in Figure 4. The influence of the density and sex ratio manipulation on CPT was statistically significant ($p = 0.01$) and resembled that observed in *Ae. aegypti* but higher than that in *Cx. quinquefasciatus*. CPT values were also lower on average across the male/female treatment combinations than those for other species. The mean CPT with 100 female:100 male mosquitoes was approximately 120 min, significantly longer than that in other treatments ($p < 0.02$). With 200 females alone, CPT with IR3535[®] declined to 48 min, as presented earlier. Results were similar for the other treatments, and none differed statistically. Based on these results, we infer that the presence of males did not strongly influence the biting frequency of the females (Figure 3).

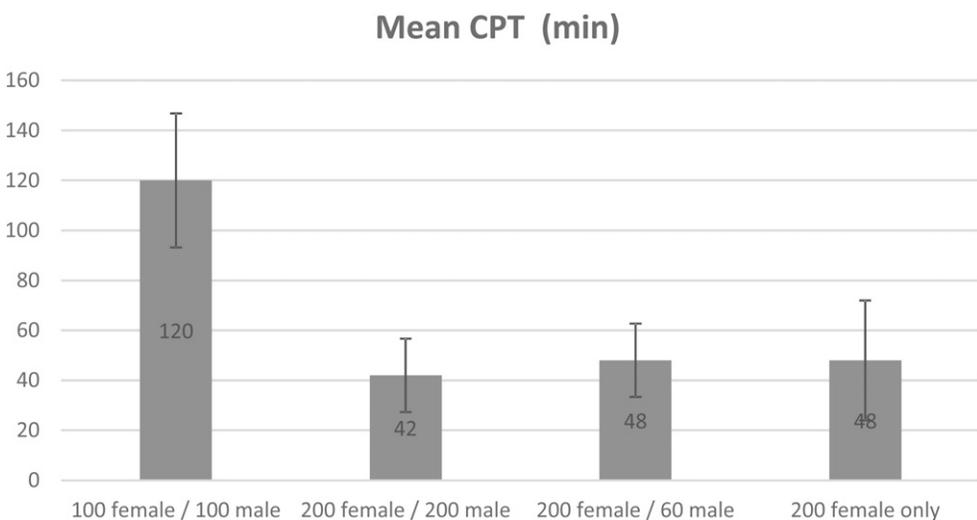


Figure 3. Repellency of 20% IR3535[®] against *Anopheles aquasalis* and determination of time to confirmed bite. The values are shown as mean CPT \pm standard deviation ($n = 5$).

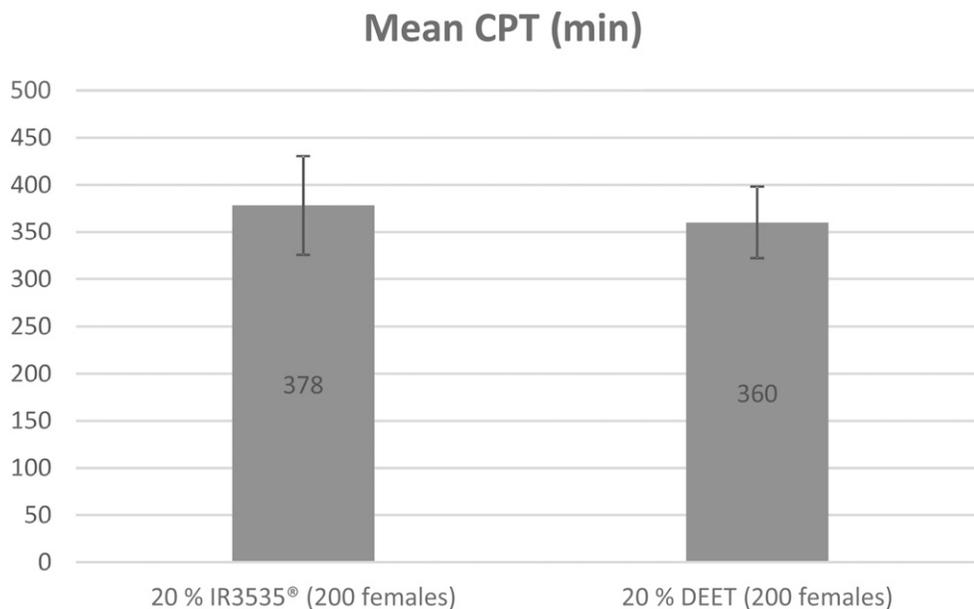


Figure 4. Repellency of 20% IR3535[®] against *Anopheles aquasalis* and determination of time to confirmed bite. The values are shown as mean CPT \pm standard deviation ($n = 5$).

REPELLENCY OF IR3535[®] AND DEET

Considering all subjects, mean CPTs across IR3535 treatments differed significantly among mosquito species ($p < 0.0001$). Mean CPT was greatest against *Cx. quinquefasciatus* (294 ± 64 min), followed by *Ae. aegypti* (197 ± 85 min) and *An. aquasalis* (65 ± 39 min). The ability of IR3535[®] and DEET to repel mosquitoes was compared using 200 females and no males *Cx. quinquefasciatus* and *An. aquasalis* females only ($N = 200$ *Cx. quinquefasciatus* or *An. aquasalis* females per cage). The outcome was principally influenced by mosquito species. Against *Cx. quinquefasciatus*, CPTs of IR3535 and DEET were 378 ± 52 and 360 ± 38 min, respectively. For repellency against *An. aquasalis*, the mean CPT was considerably less at 48 ± 25 and 72 ± 7 min for IR3535 and DEET, respectively. In neither species was the difference between repellents statistically significant (Figure 4) ($p < 0.05$).

DISCUSSION

The objective of this study was to investigate the influence of sex ratio and density of mosquitoes on the efficacy of IR3535[®] and DEET in cage tests, and to possibly add breadth to considerations of standard cage testing methodologies beyond female mosquitoes-only conditions (2). The investigation is relevant in practice because data from cage testing are used in mosquito repellent product development and to complement the field data that are required for federal registration and public health trials (1,3).

As a baseline study, we compared the efficacy of 20% IR3535[®] and 20% DEET under standard female-only conditions against *Culex quinquefasciatus* and *An. aquasalis*. Both products repelled *Culex* for a significantly longer time than the *Anopheles* mosquitoes (Figure 5).

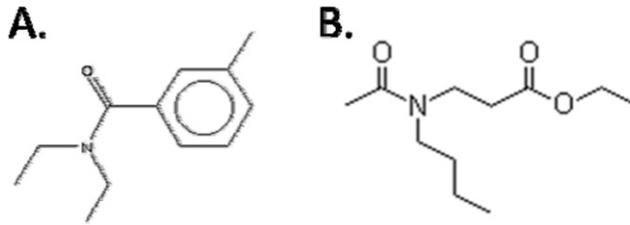


Figure 5. Structures of the repellents. (A) DEET (CAS no. 134-62-3) and (B) IR3535[®] (CAS no. 52304-36-6).

The influence of mosquito sex ratio and density on the efficacy of test chemicals was expressed as CPT for *Ae. aegypti*, *Cx. quinquefasciatus*, and *An. aquasalis*. Consistent with the baseline comparison, and regardless of the sex ratio and density, *Culex* was repelled the longest, followed by *Aedes* and *Anopheles*. Within each species, the numbers and proportions of males and females present had a marked influence on CPT.

For both *Aedes* and *Anopheles*, CPT was longest against 100 females:100 males, often being close to double than those observed for other treatments. In marked contrast, *Culex* appeared to bite more avidly in the presence of males. Across species, higher densities also appeared to reduce CPTs.

Mosquito behavior likely interacts with mosquito density and repellent action to determine protection time under the different demographic conditions (2,3,15). Mating activity by males and perhaps male-avoidance behaviors of females may reduce biting rates in *Aedes* and *Anopheles*. By contrast, in *Culex*, the presence of males or mating may serve as a cue to seek a blood meal, increasing female motivation and rendering the repellents less effective. The generally lower repellent performance when more females were present, regardless of the sex ratio, might also represent a change in female avidity due to density, or simply an increase in the absolute number of females with inherently lower thresholds for feeding in the presence of the repellent.

Differences in treatment effects observed within and among species are informative for cage testing practices and in relation to natural variation in field mosquito demography. First, cage test results can vary strikingly in response to population density, male:female ratio, and species. Second, because mosquito demography varies in space and time in nature, it is important to consider its influence in the conduct and interpretation of field test results. Although it is difficult to infer the extent to which these factors may underlie performance variation in past efficacy evaluations reported in the literature, predictions of the influence of density and sex ratio based on species-specific knowledge of mosquito feeding and mating behavior may deserve additional attention in field studies (19). In addition, knowledge of these factors may be useful in designing laboratory assays that take greater account of population variables that influence repellent performance in consumer and other public health contexts.

In addition, a long-standing focus of non-pesticidal mosquito population control is the release of large numbers of males, often at high densities. For control of disease-vectoring populations of *Ae. aegypti*, field studies (7,20) estimated the optimal density of release points for large-scale sterile male release programs to be at 50 meter intervals. Such approaches, when successful, may be self-limiting due to population declines, requiring frequent releases into the future to maintain population suppression. However, even more self-sustaining approaches, such as mass release of mosquitoes carrying multi-locus deleterious transgenes

(21), or ultimately microbes or transgenes with active drive mechanisms (21), may still strongly impact local and regional mosquito demography in ways that merit greater attention in repellent development.

CONCLUSION

Further consideration of mosquito sex ratio and density may be important for optimal development and modeling of mosquito repellent efficacy in both laboratory and field conditions. We tested the influence of mosquito density and female:male ratio in the laboratory with the topical repellent IR3535[®]. Notably, the direction of male influence in *Culex* species tested was opposite to that in *Aedes* and *Anopheles* species tested. These study results are important because they extend the study of density factors beyond DEET, and further point to striking variations among species in the outcomes of sex ratio manipulations. If confirmed, these results suggest that sex ratio and density merit greater attention in field studies, and the question of whether female-only guidelines for cage testing best model field conditions for all mosquito species should be revisited.

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